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Title of Invention: HIGH THROUGHPUT SCREEN

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. [ ] has been transmitted by the International Bureau.
  - c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
  - a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. [ ] have been transmitted by the International Bureau.
  - c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
  - d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
16. [x] Other items or information:
  - a. [ ] Verified Small Entity Statement.
  - b. [ ] Copy of Notification of Missing Requirements.
  - c. [x] Copy of cover sheet of WIPO publication (WO 99/66329) (1 sheet)

17. [X] The following fees are submitted:

**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Search Report has been prepared by the EPO or JPO.....\$860.00

International preliminary examination fee paid to

USPTO (37 CFR 1.482).....\$690.00

No international preliminary examination fee paid to

USPTO (37 CFR 1.482) but international search fee

paid to USPTO (37 CFR 1.445(a)(2)).....\$760.00

Neither international preliminary examination fee

(37 CFR 1.482) nor international search fee

(37 CFR 1.445(a)(2)) paid to USPTO.....\$1,000.00

International preliminary examination fee paid to USPTO

(37 CFR 1.482) and all claims satisfied provisions

of PCT Article 33(1)-(4).....\$ 100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT**

**= \$860.00**

Surcharge of \$130.00 for furnishing the oath or declaration later than

[ ] 20 [ ] 30 months from the earliest claimed priority date

(37 CFR 1.492(e)).

\$

Claims

Number Filed

Number Extra

Rate

Total Claims

50-20=

30

X \$18.00

\$540.00

Independent Claims

2 - 3=

X \$80.00

\$

Multiple dependent claim(s) (if applicable)

+\$270.00

\$270.00

**TOTAL OF ABOVE CALCULATIONS**

**= \$1670.00**

Reduction by 1/2 for filing by small entity, if applicable.

\$835.00

**SUBTOTAL**

**= \$835.00**

Processing fee of \$130.00 for furnishing the English translation later

than [ ] 20 [ ] 30 months from the earliest claimed priority date

(37 CFR 1.492(f)).

\$

**TOTAL NATIONAL FEE**

**= \$835.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The

assignment must be accompanied by an appropriate cover sheet

(37 CFR 3.28, 3.31).

\$40.00 per property

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a. [X] A check in the amount of **\$835.00** to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. \_\_\_\_\_ in the amount of

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The Commissioner is hereby authorized to charge any other fees due under 37 C.F.R. §1.16 or §1.17 during the pendency of this application to our Deposit Account No. 06-0916.

SEND ALL CORRESPONDENCE TO:

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7/PRTS  
High Throughput Screen

The present invention relates to a structure comprising a biological membrane and a porous or perforated substrate, a biological membrane, a substrate, a high throughput screen, methods for production of the structure membrane and substrate, and a method for screening a large number of test compounds in a short period. More particularly it relates to a structure comprising a biological membrane adhered to a porous or perforated substrate, a biological membrane capable of adhering with high resistance seals to a substrate such as perforated glass and the ability to form sheets having predominantly an ion channel or transporter of interest, a high throughput screen for determining the effect of test compounds on ion channel or transporter activity, methods for manufacture of the structure, membrane and substrate, and a method for monitoring ion channel or transporter activity in a membrane.

Within the context of this specification the word "comprises" is taken to mean "includes" and is not intended to mean "is limited to only".

Ion channels are transmembrane proteins which form pores in the membrane which allow ions to pass from one side to the other (Hille, 1992). They may show ion specificity, allowing specific ions to passively diffuse across a

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membrane down their electrochemical gradients. Although certain types of channels are on the average open all the time and at all physiological membrane potentials (so-called leak channels), many channels have 'gates' which open in response to a specific perturbation of the membrane. Perturbations known to cause opening of ion channels include a change in the electric potential across the membrane (voltage-gated channels), mechanical stimulation (mechanically-gated channels) or the binding of a signalling molecule (ligand-gated channels).

Transporters are proteins in the cell membrane which catalyse the movement of inorganic ions such as  $\text{Na}^+$  and  $\text{K}^+$  as well as organic molecules such as neurotransmitters as in the case of so-called re-uptake pumps, e.g. GABA, dopamine and glycine. Two distinguishing features of carriers versus pores are i) their kinetics-movement of ions via transporters is very much slower than the  $>10^6$  ions per second that is encountered with ion channels and ii) ion channels conduct down electrochemical gradients whereas transporters can 'pump' uphill i.e. against concentration gradients (Hille, 1992). The latter process is normally directly dependent upon energy being provided in a stoichiometric fashion.

The invention has application principally in the measurement of ion channel activity but also of transporters where these

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are electrogenic e.g.  $\text{Na}^+/\text{K}^+$ ;  $\text{Na}^+/\text{Ca}^{2+}$ ; glutamate re-uptake transporter (Brew & Attwell, 1987)

Ion channel activity has been studied using a technique referred to as "patch clamping" (Hamill et al, 1981).

5 According to this technique a small patch of cell membrane is generally isolated on the tip of a micropipette by pressing the tip against the membrane. It has been suggested that if a tight seal between the micropipette and the patch of membrane is established electric current may  
10 pass through the micropipette only via ion channels in the patch of membrane. If this is achieved the activity of the ion channels and their effect on membrane potential, resistance and current may be monitored. If the electric potential across the membrane remains constant the current  
15 supplied to it is equal to the current flowing through ion channels in the membrane. If ion channels in the membrane close resistance of the membrane increases. If the current applied remains constant the increase of resistance is in direct proportion to an increase of electric potential  
20 across the membrane.

Many drugs are known to exert their effect by modulation of ion channels, but the development of novel compounds acting on them is hampered considerably by the difficulty of screening at high-throughput rates for activity.

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Conventional electrophysiological methods such as patch or voltage clamp techniques provide definitive mechanistic information but suffer from the problem that they are unsuited to the rapid screening of test compounds.

5 WO96/13721 describes apparatus for carrying out a patch clamp technique utilized in studying the effect of certain materials on ion transfer channels in biological tissue. It discloses patch clamp apparatus utilizing an autosampler, such as those utilized with HPLC apparatus, to provide a  
10 higher throughput than may be achieved by the conventional patch clamp technique. This apparatus suffers from the problems that it merely semi-automates the drug delivery system, not the patch clamp recording. It therefore suffers from the same limitations as traditional patch-clamping with  
15 respect to speed of processing compounds and can in no way be considered a high-throughput system. The system still requires linear processing (i.e. processing of data obtained for one cell after another). In direct contrast the invention described herein provides parallel processing and  
20 thus genuine high-throughput of compounds.

The term "biological membrane" used herein is taken to include artificial membranes such as lipid bilayers and other membranes known to a person skilled in the art.

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The present invention addresses the problems associated with the known screens and screening methods.

In a first aspect the present invention provides a structure which comprises a biological membrane adhered with a high resistance seal to a porous or perforated substrate for use  
5 in a high throughput screen wherein the biological membrane comprises an ion channel or transporter.

In a second aspect the invention provides a biological membrane for use in the structure which is capable of  
10 adhering to a substrate with a high resistance seal wherein each cell forms a tight junction with adjacent cells and expresses an ion channel which is localised in the cell membrane.

In a third aspect the invention provides a substrate for use  
15 in a high throughput screen which is perforated.

In a fourth aspect the invention provides a high throughput (HiT) screen for the detection and assay of test compounds with activity on voltage gated ion channels which comprises the biological membrane.

20 In a fifth aspect the invention provides a method of manufacturing a structure comprising a biological membrane adhered with a high resistance seal to a perforated

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substrate which comprises the steps of selecting a substrate, perforating it, introducing a biological membrane to the substrate and sealing each pore with biological membrane.

- 5 In a sixth aspect the invention provides a method of manufacturing the biological membrane which comprises the steps of selecting a cell type, evaluating it for ability to form contiguous layers of cells with tight junctions and for low to negligible numbers of voltage gated ion channels, 10 culturing the cells on a substrate and ensuring that a contiguous layer of cells is grown.

- In a seventh aspect the invention provides a method of manufacturing a perforated substrate which comprises the steps of shining a laser of preselected focal area, power or 15 time of exposure at a coverslip to perforate it. This method also may include the additional step of modification of the perforated area by exposure to plasma and/or localised heating in order to attain the appropriate level of smoothness of the perforation(s).

- 20 In an eighth aspect the invention provides a method of screening for the detection or assay of compounds with activity on ion channels which comprises the steps of placing a biological membrane which expresses ion channels of interest in contact with test compound in physiological



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solution or non-physiological solution comprising a solvent such as dimethyl sulphoxide and measuring the resistance or impedance of the biological membrane under the influence of test compound.

5 Preferably an embodiment of the biological membrane comprises cells having an ion channel or transporter which naturally resides in the cell membrane thereof, or it can be inserted by transfection with cDNA and/or cRNA encoding the ion channel or transporter. The invention thus has the  
10 advantage that it permits studies of native channels or transporters where the precise subunit composition is unknown or indeed where the molecular identity is completely unknown (i.e. not yet cloned) but also heterologously-expressed cloned channels or transporters where the identity  
15 of the native channel or transporter is known or where precise knowledge is required of the interaction of compound structures and chemical moieties of the ion channel or transporter. Therefore the system is substantially more versatile than existing approaches which are insensitive and  
20 rely on getting high concentrations of cells (not always possible with neurones) and high signal to noise ratios which limits their use to only certain types of cells and ion channels.

Preferably an embodiment of the biological membrane  
25 comprises a plurality of ion channels or transporters which

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are predominantly preselected ion channels or transporters of interest. This provides the invention with the advantage of permitting parallel screening of different channels potentially providing an even higher throughput of compounds.

More preferably an embodiment of the biological membrane comprises genetically engineered cells which have been engineered to predominantly express an ion channel or transporter.

10 Preferably the ion channels are voltage gated ion channels.

Preferably an embodiment of the biological membrane comprises cells selected from the group which comprises HEK-293 cells, genetically modified Chinese hamster ovary (CHO) cells, primary neuronal tissue such as hippocampus, dorsal root ganglia, superior cervical ganglia etc.; skeletal muscle; smooth muscle; cardiac muscle; immune cells; epithelia; endothelia etc.

CHO cells and CHO sub-clones such as CHO-K1 and CHO-dhfr (also known as Dukx) have exceptionally low levels of endogenous ion channel expression thus providing the advantage of having excellent signal to noise characteristics within a mammalian cell environment. Similarly, HEK-293 (human embryonic kidney) cells express

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low levels of native channels and provide a human expression 'background'. Both these expression systems are infinitely preferable to the well-used *Xenopus* oocyte technique where not only are native channels and subunits abundant, but the  
5 amphibian cell environment differs in important ways from mammalian cells.

Preferably an embodiment of the biological membrane comprises ion channels having rapid activation and inactivation kinetics which existing methods of high-  
10 throughput screening can not resolve. Existing systems, therefore, average transient ion channel signals frequently over periods of many seconds. Channels inactivating with time-constants of the order of milliseconds and without a steady-state presence are effectively undetectable in such  
15 systems. The invention presented here however, has the advantage that it can easily resolve such kinetics just as traditional patch clamping does, but at high-throughput rates.

Preferably an embodiment of the biological membrane  
20 comprises ion channels which show specificity for ions selected from the group which comprises sodium, potassium, calcium, chloride.

Preferably an embodiment of the biological membrane comprises a contiguous layer of cells capable of adhering

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with a high resistance seal to substrates selected from the group which comprises perforated glass, plastics, rubber, polytetrafluoroethylene (PTFE), PTFE/glass fabric and polyethylene terephthalate (PETP).

5 Preferably an embodiment of the biological membrane comprises a pseudo-epithelium wherein one face of a contiguous layer of cells is permeabilized thereby providing access to the interior of the cells. This has the great advantage of providing the means for current and voltage-clamping which is not possible with any existing high-throughput screening system. Not only does this permit high time-resolution recording but it also provides the means to stimulate or activate voltage-gated ion channels in a precise and controlled manner. For example, it is not  
10 necessary to alter the ionic composition e.g. by elevating  $K^+$  to depolarize cells, which in itself can modulate the kinetics of ion channels (e.g.  $K^+$  channels) and also obscure the activity of novel ligands by competition at ion channel binding sites. This is a very great advantage over all  
15 existing systems. Permeabilization also allows the introduction to the cytosol of compounds that otherwise could not do so either by virtue of molecular weight or physicochemical characteristics.

20

Preferably an embodiment of the biological membrane  
25 comprises a contiguous layer of cells which is permeabilized

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by an antibiotic selected from the group which comprises amphotericin and nystatin; or detergent selected from the group which comprises digitonin and saponin; or physical disruption using a high voltage field; or by enzymatic digestion of a part of the membrane using an appropriate enzyme.

An advantage of using high voltage fields to permeabilize the membrane (electropermeabilisation) is that such a technique can permeabilize the plasmamembrane while sparing smaller intracellular structures such as mitochondria and endoplasmic reticulum. The technique can also be controlled very precisely and would not necessitate a facility to exchange solutions in a lower chamber of the recording apparatus.

Preferably an embodiment of the substrate comprises a perforated coverslip.

Preferably an embodiment of the substrate has pores of diameters between  $0.5\mu\text{m}$  and  $10\mu\text{m}$ . More preferably the pores are of diameters between  $1\mu\text{m}$  and  $7\mu\text{m}$ . More preferably the diameter is  $1-2\mu\text{m}$ .

Preferably an embodiment of the substrate comprises a grid of pores of greater number than 4 but less than 10. This provides the advantage of a statistically acceptable number

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of parallel recordings (i.e.  $\geq 4$ ) in each treatment but small enough that the ratio of pores to cells can be made vanishingly small and thus the probability that a pore is sealed with and therefore occluded by a cell extremely high.

5 Preferably an embodiment of the substrate according to the invention is manufactured of a material selected from the group which comprises glass, plastics, rubber, polytetrafluorotethylene (PTFE), PTFE/glass fabric and polyethylene terephthalate (PETP).

10 Preferably an embodiment of the screen comprises:

a plurality of chambers, each having a permeable peripheral surface providing a substrate for the biological membrane;

15 a plurality of wells each capable of receiving a chamber and a test compound in a physiological solution or non-physiological solution comprising dimethyl sulfoxide (DMSO) or other solvent;

a plurality of reference electrodes, at least one having electrical contact with each well;

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a movable recording head carrying at least one recording electrode thus providing the basic requirement for automated recording of ion channel activity in a multiwell plate format;

5 means for measuring electrical resistance or impedance between the recording and reference electrodes; wherein

electrical current may pass between the recording and reference electrodes through the permeable peripheral surface of each chamber only via ion channels or  
10 transporters in the biological membrane.

Preferably an embodiment of the screen comprises wells which are provided by a multiwell plate. The advantage of this being that high throughput can be achieved using industry-standard components which can be processed using  
15 commercially available automated equipment and robotics. Users will have the possibility of using their existing plate processing equipment thus containing costs in establishing a state-of-the-art high-throughput electrophysiology screen.

20 Preferably an embodiment of the screen comprises a perforated substrate for the biological membrane.

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Preferably a further embodiment of the screen comprises a structure or biological membrane described above having ion channels of interest in an array of droplets on a porous substrate. Preferably an array of miniature recording chambers is created by placing a 'lid' incorporating recording electrodes over the matrix of droplets such that a meniscus of the droplet solution is established. Preferably a test compound in electrically conducting solution is placed in at least one of the droplets or applied via access ports in the 'lid' and the resistance/impedance (in current-clamp configuration) of the biological membrane or conductance (in voltage-clamp configuration) is measured under the influence of the test compound. An advantage of this approach is that sheets of substrate can be designed without the need to transfer pieces of substrate (e.g. discs) to multiwell plates and also obviates complex chamber design with seals, 'O'-rings and the like. The invention can still accommodate addition of solutions and has an additional advantage of using very small volumes and thus small quantities of reagents and cells. Excellent insulation is afforded by the air gaps between adjacent droplets.

Preferably an embodiment of the recording head comprises a single recording electrode capable of being moved to visit each chamber sequentially. More preferably an embodiment of the recording head comprises a plurality of recording



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electrodes arranged in a line. Even more preferably the recording head comprises a plurality of recording electrodes arranged in a matrix. The advantage of this configuration is that simultaneous recording from all wells is possible  
5 via a data-acquisition multiplexing system.

Preferably an embodiment of the screen is capable of multiplexing up to 384 recording elements to a data acquisition system utilizing multiple voltage-clamp amplifiers. This has the advantage of providing extremely  
10 high time resolution and effectively simultaneous measurement from all wells. This has the advantage of providing the TERM system with the potential to achieve throughput rates similar to the best possible for conventional fluorescence-based ligand-receptor binding  
15 assays ( $\geq 150,000$  compounds per week).

Preferably an embodiment of the method of manufacturing the structure comprises the steps of simultaneously perforating a coverslip and sealing the pores with biological membrane. This embodiment provides the advantage of eliminating steps  
20 in the establishment of the final configuration, namely procedures required to optimise the probability of a cell sealing with pores in the perforated substrate. This has the advantage of simplifying the final product.

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Preferably an embodiment of the method of manufacturing the biological membrane includes the step of permeabilizing one surface of the contiguous layer of cells thereby providing access to the interior of the cells. This has the great advantage of providing the means for current and voltage-clamping which is not possible with any existing high-throughput screening system. Not only does this permit high time-resolution recording but is also provides the means to stimulate or activate voltage-gated ion channels in a precise and controlled manner. For example, it is not necessary to alter the ionic composition e.g. by elevating  $K^+$  to depolarize cells, which in itself can modulate the kinetics of ion channels (e.g.  $K^+$  channels) and also obscure the activity of novel ligands by competition at ion channel binding sites. This is a very great advantage over all existing systems. Permeabilization also allows the introduction to the cytosol of compounds that otherwise could not do so either by virtue of molecular weight or physicochemical characteristics.

Preferably the permeabilization is carried out by the step of contacting the surface with an antibiotic selected from the group which comprises amphotericin and nystatin; or detergent selected from the group which comprises digitonin and saponin; or physical disruption using a high voltage field; or by enzymatic digestion of a part of the cell membrane using an appropriate enzyme.

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An advantage of using high voltage fields to permeabilize the membrane (electropermeabilisation) is that such a technique can permeabilize the plasmamembrane while sparing smaller intracellular structures such as mitochondria and endoplasmic reticulum. The technique can also be controlled very precisely and would not necessitate a facility to exchange solutions in a lower chamber of the recording apparatus.

Preferably an embodiment of the method of manufacturing the biological membrane includes the steps of transfecting cells with cDNA or cRNA encoding an ion channel of interest and cloning cells expressing the ion channel of interest. These steps provide the invention with the advantage of permitting studies of heterologously expressed cloned channels where the identity of the native channel is known or where precise knowledge is required of the interaction of compound structures and chemical moieties of the ion channel.

Preferably an embodiment of the method of manufacturing the perforated substrate comprises the steps of adjusting the profile, taper or diameter of the pore with a laser.

Preferably the laser source is controlled by an automated stage under control of a computer and inverted phase-contrast microscope which provides the advantage of

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permitting visual examination of the pore characteristics  
e.g. profile, taper and diameter.

Preferably an embodiment of the method of manufacturing the  
perforated substrate comprises other non-laser methods such  
5 as photo-etching, casting and physical piercing of the  
substrate.

Preferably an embodiment of the screening method comprises  
the step of measuring ion channel activity by monitoring  
trans-epithelial resistance measurements (TERM) across an  
10 intact cell layer.

In a further embodiment of the screening method a surface of  
the contiguous cell layer is preferably permeabilized  
thereby providing access to the interior of the cells. This  
has the great advantage of providing the means for current  
15 and voltage-clamping which is not possible with any existing  
high-throughput screening system. Not only does this permit  
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K<sup>+</sup> to depolarize cells, which in itself can modulate the  
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the activity of novel ligands by competition at ion channel  
binding sites. This is a very great advantage over all

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existing systems. Permeabilization also allows the introduction to the cytosol of compounds that otherwise could not do so either by virtue of molecular weight or physicochemical characteristics.

5 Preferably a surface of the contiguous cell layer is permeabilized by antibiotics selected from the group which comprises amphotericin and nystatin; or detergents selected from the group which comprises digitonin and saponin; or physical disruption using a high voltage field; or by  
10 enzymatic digestion of a part of the membrane using an appropriate enzyme thereby permitting intracellular voltage or current measurements to be made.

An advantage of using high voltage fields to permeabilize the membrane (electropermeabilisation) is that such a  
15 technique can permeabilize the plasmamembrane while sparing smaller intracellular structures such as mitochondria and endoplasmic reticulum. The technique can also be controlled very precisely and does not necessitate a facility to exchange solutions in a lower chamber of the recording  
20 apparatus.

Preferably an embodiment of the invention provides a screening method which includes the step of multiplexing up to 384 recording elements to a data acquisition system utilizing multiple voltage-clamp amplifiers. This has the

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advantage of providing extremely high time resolution and effectively simultaneous measurement from all wells. This has the advantage of providing the TERM system with the potential to achieve throughput rates similar to the best possible for conventional fluorescence-based ligand-receptor binding assays ( $\geq 150,000$  compounds per week).

Preferably an embodiment of the method of screening for the detection or assay of compounds with activity on ion channels of interest in an array of droplets on a porous substrate. An array of miniature recording chambers may be created by placing a 'lid' incorporating recording electrodes over the matrix of droplets such that a meniscus of droplet solution is established. A test compound in conducting solution is placed in at least one of the droplets or applied via access ports in the 'lid' and the resistance of the biological membrane or conductance (in voltage-clamp configuration) is measured under the influence of the test compound.

In an alternative embodiment of the screening method the biological membrane is placed in a plurality of chambers and test compound in physiological solution, or non-physiological solution comprising a solvent eg dimethyl sulphoxide, is added to the chambers.

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Preferably an embodiment of the screening method comprises the steps of drug delivery and washing of the multi-well plate.

Preferably an embodiment of the screening method  
5 incorporates a step of stimulation of cells involving the use of a photoactivatable 'ion scavenger' eg of ions such as  $K^+$ . The active entity can be released by flashing the entire plate at once with a high intensity light source eg a laser or Xenon lamp. The advantage of this system is that  
10 membrane potential can be altered by altering the ionic distribution in a non-invasive fashion and with fine temporal control.

It has surprisingly been found that a biological membrane can be adhered with a high resistance seal to a perforated  
15 substrate for use in a high throughput screen for test compounds having activity on ion channels. This was considered unobvious to a person skilled in the art at the outset in view of the fact that achievement of a high resistance seal has not been possible without an undue  
20 burden. Furthermore, perforated substrates having a biological membrane sealed thereto have not been suggested for use in high throughput screens.

It has surprisingly been found that a biological membrane capable of adhering with a high resistance seal to a

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substrate may be constructed for use in a high throughput screen. Surprisingly it has been found that the biological membrane may be constructed having ion channels which are predominantly the ion channels of interest. Furthermore, it  
5 has surprisingly been found that a high throughput screen may be constructed and used to detect and assay a throughput of test compounds which may be in excess of 30000 per week.

Surprisingly the screen may be used to obtain bona fide electro physiological data relating to functional ion  
10 channel activity.

The biological membrane of the invention was unobvious at the outset to a person skilled in the art. Construction of a biological membrane having high resistance seals with a substrate such as perforated glass had not been achieved and  
15 was not considered possible without an undue burden. In addition construction of a membrane having ion channels which are predominantly an ion channel of interest had not been considered possible without an undue burden.

The high throughput screens and methods of the invention  
20 were unobvious at the outset to a person skilled in the art in view of the fact that it was not considered possible without an undue burden to screen the high throughput of test compounds which may be achieved by the invention.



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In addition to the advantage of a high-throughput of test compounds, embodiments of the screen and method of the invention may provide functional assays (cf. say ligand binding) in which the mode of action (eg. blocking or enhancing) of the test compound on voltage gated ion channels is measured via changes in membrane resistance or by recording the current flowing through ion channels in the membrane directly.

The invention will now be described by reference to the following examples of preferred embodiments and accompanying drawings in which:

Figure 1 shows an epithelial cell version of a screen according to an embodiment of the invention.

Figure 2 shows an embodiment of the screen of the invention having a perforated substrate.

Figure 3 shows adaption of a commercially available multi-well plate for use in a screen according to an embodiment of the invention. The figure shows an integral multi-recording electrode head cluster.

Figure 4 shows an embodiment using a movable recording head wherein a single recording head reads single wells sequentially.

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Figure 5 shows an embodiment of a fluid matrix system wherein an array of miniature recording chambers are created by dispensing droplets on to the recording substrate in a pre-determined pattern and density. Figure 5 (f) shows the full sandwich (recording configuration) of the system.

Figure 6 shows a further embodiment of a fluid matrix system wherein multiple arrays of droplets are sandwiched together.

Figure 7 shows a pore formed in a substrate, according to the invention. The light micrograph shows a pore in a thin glass substrate. The pore, which was approximately 2 micrometers in diameter, was manufactured by using pulses of focussed laser energy followed by a combination of fire polishing and plasma modification. The scale bar is 10 micrometers across.

## 15 Examples

An embodiment of the screen of the invention comprises a multi well plate with integrated recording devices, by which means a massively parallel voltage clamp (MPVC) can be performed on a plurality of wells in a plate within short periods of time (ca. 1-60s). Preferably commercially available 96 or 384 well plates are employed, or the 96 or 384 position array format is adopted.

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An embodiment of the screen of the invention preferably provides a throughput of test compounds in excess of 30,000 per week with bona fide electrophysiological 'read-out' of functional ion channel activity. An embodiment of the screen may provide high resolution both in terms of time; for a 96 well plate, 1ms/point/voltage clamp setup. The sensitivity to modulation of channel activity is  $\geq 1\%$ .

An embodiment of the present invention provides a method which comprises the steps of measuring transepithelial electrical resistance across a contiguous layer of cells expressing predominantly an ion channel of interest. It will be apparent to a person skilled in the art that the method depends on adherence of the cells to a substrate that allows formation of tight junctions between adjacent cells such that a high-resistance sheet is formed. Changes in activity of the ion channels expressed in the membranes of individual cells are reflected by changes in the resistance across the sheet as a whole. In a refinement of this embodiment of the invention, access to the interior of the cells comprising the sheet is obtained by means which permit population current and voltage clamp recording to be made.

Transepithelial resistance measurements have been carried out as follows:

a) Epithelial/ endothelial type cells.

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The overall transepithelial resistance is composed of two principal components, the sum of the 'whole-cell' conductances of the individual cells making-up the pseudo-epithelium and the sum of the intercellular conductance pathways.

Naturally-occurring epithelial (and endothelial) cells form tight junctions with one another. This tight packing reduces the leakage of ions/ solutes between cells, resulting in relatively low numbers of intercellular conductance pathways across the membrane as a whole. Thus, where conditions permit tight junctions to form, changes in the cell transmembrane conductance are measureable. One approach to this method was to select a host cell with appropriate growth properties. Cell-types which also express native ion channels at low levels are considered suitable for expression of cloned ion channels. A large hurdle for a person skilled in the art in this approach lay in obtaining cells in which the latter is true.

b) Transepithelial resistance measurement in non-epithelial cells.

An alternative to the approach described above is to use non-epithelial cells that are known to express negligible numbers of ion channels of their own as a basic expression system for cloned cells which express ion channels of

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choice. There are several cell-types that fulfill this criterion. However, a large hurdle to a person skilled in the art was presented in that they do not form contiguous layers of cells in culture with tight junctions. In an  
5 embodiment of the invention there is provided a high resistance 'epithelial' layer in which this hurdle has been overcome.

#### Transepithelial current measurement (massively parallel voltage clamp (MPVC))

10 An embodiment of the invention was obtained by gaining access to the interior of cells in the 'epithelium' by disrupting or permeabilizing one face of each cell contributing to the cell layer. This has been carried out by chemical methods, for example by allowing certain types  
15 of antibiotics (eg. amphotericin) or detergents (digitonin) to come into contact with one face of the cell surface or through physical disruption eg. using high voltage fields (electroporation/ integral zipper).

#### Electrical recording Systems

20 A number of systems have been developed and they are outlined below:

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### Pilot test systems

For pilot testing of the integrity of pseudo-epithelial layers, transepithelial resistance was measured using a chamber into which permeable tissue culture supports were inserted (Fig 1). Cells were grown in culture to confluency on this support. In the case of perforated substrates, the material (eg. coverslip) was inserted in a purpose-built test rig which permitted variation in the pressure in the lower compartment and/ or upper chamber and at the same time allowed resistance measurements to be made (Fig 2). To avoid polarization of the electrodes, an oscillating voltage signal was passed across the cell-layer via linear or circular electrodes positioned above and below the permeable support on which the layer of cells was growing and the impedance of the cell-layer was measured. In the case of permeabilized cell-layers (Figures 1b and 2b), voltage and current-clamp recording was carried out using a voltage-clamp amplifier interfaced with a computer to control experimental protocols and sample the data.

### Scale-ups and operational systems

In either TERM or MPVC commercial screens utilize a multiwell plate measuring system (Fig 3) or equivalent (eg. using a droplet matrix generated using a nano litre piezo-dispenser). This was derived to some extent from the

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pilot test rig but required the design of an integral recording head of which embodiments of the invention include a number of possibilities. They are described below.

5 i) single recording head which reads single wells sequentially (Figure 4).

ii) moveable linear row of recording heads (eg. 12 for a 96 well plate system; 24 for a 384 well system) which are moved across the plate in 8 (96 well) or 16 (384 well) steps.

10 iii) electrode matrix built into the plate with multiplexing for recording headstage & acquisition system. For larger density plates multiple voltage-clamps were used to maintain sampling frequency and therefore time resolution (Figure 3).

iv) droplet system (Figure 5).

#### Multiwell plate adaptation

15 Embodiments of the screen of the present invention preferably include an integral automated pipettor in the working versions of TERM and MPVC.

20 Preferably embodiments of the screen of the invention include a facility for washing recording heads between use in separate rows.

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According to an embodiment of the invention the method of manufacture of the biological membrane comprises the steps of obtaining a high resistance seal with perforated glass substrate (or other support) and/ or the step of obtaining  
5 a cell-line having the ability to form sheets and having low or negligible numbers of native ion channels.

#### Epithelial cell approach

Naturally occurring cell-lines and engineered cell-lines have been developed. They are described below:

#### 10 Naturally-occurring cell-lines

Cell-lines referred to in the literature have been evaluated for 'off the shelf' suitability. Initial candidates included ECV-304, RBE4 and C6 glioma cells. Criteria for use were:

a) ability to form contiguous layers of cells with tight  
15 junctions; transepithelial resistance of  $\geq 125 \Omega \text{cm}^{-2}$ .

b) low to negligible numbers of background voltage-gated ion channels as assessed by whole cell patch clamp by standard methods. Preferably the conductance level is  $\leq 2 \text{nS}$  per cell.



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### Engineered cell-lines

A suitable cell-line was prepared by molecular engineering. If background conductances of the naturally-occurring cell-lines were above the threshold given in (b) above, the  
5 cell-line was assessed for possible gene knock-out to engineer a novel host cell.

### Artificial epithelia

#### Perforated substrates

Perforated substrates have been developed as set out below:

#### 10 Laser-generated substrates

##### a) Prototypes

Glass coverslips were perforated in a sequential fashion (1 hole at a time) using a laser energy source in conjunction with automated stage under computer control and an inverted  
15 optics microscope. This permitted prototypes to be constructed with fine control of parameters such as focal area, laser power and time of exposure. The ability to achieve high resistance sealing between cells and substrate was tested using coverslips created in this way.

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Grid patterns were reproducibly generated in variable formats by means of a computer-controlled stage which was interfaced with the laser via the computer. Coverslips of various materials including glass (as well as plastics and other biocompatible materials) were used. Their diameters were 10mm (96 well plate) or 5mm (384 well plate); and of variable thickness (ca.1-20 $\mu$ m). Pores were generated with diameters varying between 0.5 $\mu$  and 10 $\mu$ . The profile of the pore, its taper and internal and external diameters were evaluated to optimise sealing with the test cells. It is important to establish the appropriate level of smoothness of the pore. Pore density was optimized for signal-to-noise characteristics, fidelity of voltage-clamp recording and statistical considerations.

To encourage sealing between cell and pore, a number of approaches were taken (Fig 1). They are outlined below:

i) negative pressure in lower liquid compartment eg. using a venturi effect caused by flowing solution across the ventral orifice and/or by supplying the flowing solution at a reduced overall pressure.

ii) positive pressure in the upper liquid compartment

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iii) coating of the coverslip with anti-adhesion material that is burned off in the pore region during the pore manufacturing process (ie. laser induced pore formation)

iv) facility to jog or vibrate coverslip to encourage cells  
5 to 'find' pores before adhering to the substrate at non-pore locations

v) either a coverslip carousel or multiwell plate carousel to permit centrifugation.

vi) application of voltage field across pores to move cells  
10 into pore mouth.

Surprisingly, laser induced pore formation provided remarkable results.

Figure 7 shows a typical pore produced by this method. When physiological solutions were added to either side of the  
15 pore, trans-substrate resistances, typically in the range 200 kOhms to 400 kOhms, were routinely observed. With the addition of cells, the observed resistance was approximately double this figure. With the additional application of one or more of the approaches outlined above, resistance  
20 measurements approaching the gigaohm range were observed.

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### b) Scale-ups

Bulk perforation and simultaneous recording (sealing) were evaluated. The approach comprised 'flashing' the whole bottom surface of a multiwell plate (or equivalent matrix) with a high energy laser source. With appropriate well structure, the precise location of the required pores was known and with appropriate titration of cell density, a high probability of a having a cell 'in residence' was achieved. The plate was perforated and the ventral cell surface breached almost simultaneously. This required a much higher energy laser than that used in prototypes (above).

### c) Cell-types

Although Chinese hamster ovary (CHO) cells have been used to develop the invention, it will be apparent to a person of ordinary skill in the art that a wide variety of cell-lines and primary cells such as neurones isolated from intact tissues may be employed.

### Other perforation methods

Alternative methods of perforating glass coverslips and other materials have been evaluated such as etching, casting glass or plastics sheets.

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### Porous rubber

Porous rubber substrates are commercially available for growing cells in cell-culture. The porosity has been evaluated in the context of the resistance and current-measuring applications described herein.

### Other materials

It will be apparent to a person skilled in the art that additional materials such as PTFE, PETP etc. may be employed in accordance with the present invention. These have the advantage of having high dielectric constants but also of being manufactured in extremely thin sheets. This has the advantage of reducing the minimum series resistance in the whole system and also facilitating the introduction of exogenous substances to the cell cytosol.

### Multi-well plate recording apparatus

The basal multi-well plate recording apparatus preferably accommodates a 96-well/ location format. Preferably multiples of the 96-well format are constructed with a minimal expansion to 384 well format. An advantage of increasing well-density is that the amount of test compound used is reduced and fewer plates are used with concomitant reductions in ancillary running costs per compound tested.

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The following two approaches have been evaluated:-

a) A TORM workstation designed to interface with commercially-available robots and plate processors.

b) Fully integrated stand-alone system which provides plate  
5 handling, solution changes and recording headstages.

#### Fluid Matrix System

An array of miniature recording chambers were created by dispensing droplets containing a suspension of cells onto the recording substrate in a pre-determined pattern and density (see Figure 5). The substrate can be of any of the  
10 types described above eg. perforated glass, plastic, rubber, etc. The complete recording configuration is accomplished by placing a 'lid', or moveable recording head, incorporating recording electrodes over the matrix of  
15 droplets such that a meniscus of the droplet solution is established as exemplified in Fig 5. An array of droplets may also be generated on the reverse of the porous substrate to provide a conducting pathway to at least one reference electrode and also the means by which substances may be  
20 applied to the lower surface of the substrate and hence cell membranes. Similarly, reagents can be applied via a further droplet matrix applied to the 'recording plate' as shown in Figure 6. Drug solutions may be dispensed onto a recording

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"head" plate; the plate may then be inverted; and the inverted plate may then be docked with the cell matrix to bring the drug into contact with the cells. The advantage of this approach is that sheets of substrate can be designed  
5 without the need to transfer substrate discs to multiwell plates and also obviates complex chamber design with seals, 'O' -rings and the like. The invention can still accommodate addition of solutions and has the additional advantage of using very small volumes and thus small  
10 quantities of reagents and cells.

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## Figure Legends

- |    |    |   |   |
|----|----|---|---|
|    | 1  | : | cells                                   |
|    | 2  | : | permeabilized cell surface              |
|    | 3  | : | voltage clamp                           |
| 5  | 4  | : | porous substrate                        |
|    | 5  | : | electrode                               |
|    | 6  | : | well wall                               |
|    | 7  | : | solution perfusion channel              |
|    | 8  | : | cell line or primary cell               |
| 10 | 9  | : | permeabilized cell                      |
|    | 10 | : | multiwell plate (e.g. 96 wells)         |
|    | 11 | : | integral recording head cluster         |
|    | 12 | : | multiplexer                             |
|    | 13 | : | ADC/computer                            |
| 15 | 14 | : | fluid level                             |
|    | 15 | : | pore                                    |
|    | 16 | : | O-ring                                  |
|    | 17 | : | recording assembly                      |
|    | 18 | : | cell plate                              |
| 20 | 19 | : | reference plate                         |
|    | 20 | : | recording plate                         |
|    | 21 | : | reference electrode                     |
|    | 22 | : | recording electrode                     |
|    | 23 | : | "demi-sandwich"                         |
| 25 | 24 | : | full sandwich (recording configuration) |

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## Claims

1. A structure which comprises a biological membrane adhered with a high resistance seal to a porous or perforated substrate for use in a high throughput screen wherein the biological membrane comprises an ion channel or transporter.  
5
2. A structure according to claim 1 wherein the biological membrane comprises a contiguous layer of cells which is capable of adhering to a substrate with a high resistance seal wherein each cell forms a tight junction with adjacent cells and expresses an ion channel or transporter which is localised in the cell membrane.  
10
3. A structure according to claim 1 or 2 which comprises cells having an ion channel or transporter which naturally resides in the cell membrane thereof or, it can be inserted by transfection with cDNA and/or cRNA encoding the ion channel or transporter.  
15
4. A structure according to any preceding claim which comprises a plurality of ions channels or transporters which are predominantly preselected ion channels or transporters of interest.  
20

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5. A structure according to any preceding claim which comprises genetically engineered cells which have been engineered to predominantly express an ion channel or transporter.
- 5 6. A structure according to any preceding claim which comprises voltage gated ion channels.
7. A structure according to any one of claims 2 to 5 wherein the cells are selected from the group which comprises HEK-293 cells, genetically modified Chinese hamster ovary (CHO) cells, primary neuronal tissue such as hippocampus, dorsal root ganglia, superior cervical ganglia etc.; skeletal muscle; smooth muscle; cardiac muscle; immune cells; epithelia; endothelia.
- 10 8. A structure according to any preceding claim which comprises an ion channel having rapid activation and inactivation kinetics.
- 15 9. A structure according to any preceding claim having an ion channel which shows specificity for an ion selected from the group which comprises sodium, potassium, calcium, chloride.
- 20 10. A structure according to any one of claims 2 to 9 wherein the contiguous layer of cells is capable of

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adhering with a high resistance seal to a substrate selected from the group which comprises glass, plastics, rubber, polytetrafluoroethylene (PTFE), PTFE/glass fabric and polyethylene terephthalate (PETP).

- 5
11. A structure according to any preceding claim which comprises a pseudo-epithelium wherein one face of a contiguous layer of cells is permeabilized thereby providing access to the interior of the cells.
- 10 12. A structure according to claim 11 which comprises a pseudo-epithelium wherein one face of the contiguous layer of cells is permeabilized by an antibiotic selected from the group which comprises amphotericin and nystatin; or detergent selected from the group
- 15 which comprises digitonin and saponin; or physical disruption using a high voltage field; or by enzymatic digestion of a part of the membrane using an appropriate enzyme.
- 20 13. A structure according to any preceding claim wherein the substrate is perforated.
14. A structure according to any preceding claim which comprises a perforated coverslip.

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15. A structure according to any preceding claim wherein the substrate has pores of diameters between  $0.5\mu\text{m}$  and  $10\mu\text{m}$ .
16. A structure according to claim 15 wherein the pores are  
5 of diameter between  $1\mu\text{m}$  and  $7\mu\text{m}$ .
17. A structure according to claim 15 or 16 wherein the pores are of diameter  $1-2\mu\text{m}$ .
18. A structure according to any preceding claim which comprises a coverslip having a grid of pores.
- 10 19. A structure according to any preceding claim which comprises a perforated substrate which is manufactured of a material selected from the group which comprises glass, plastics, rubber, polytetrafluoroethylene (PTFE), PTFE/glass fabric and polyethylene terephthalate (PETP).  
15
20. A biological membrane for use in the structure according to any preceding claim.
21. A substrate for use in the structure according to any one of claims 1 to 19.

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22. A high throughput screen for detecting and assaying compounds with activity on voltage gated ions channels which comprises a structure according to any one of claims 1 to 19, a biological membrane according to any claim 20 or a substrate according to claim 21.

23. A high throughput screen according to claim 22 which comprises :

a plurality of chambers, each having a permeable peripheral surface providing a substrate for the biological membrane;

a plurality of wells each capable of receiving a chamber and a test compound in a physiological solution or non-physiological solution comprising dimethyl sulphoxide;

a plurality of reference electrodes, having electrical contact with each well;

a movable recording head carrying at least one recording electrode;

means for measuring electrical resistance or impedance between the recording and reference electrodes; wherein

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electrical current may pass between the recording and reference electrodes through the permeable peripheral surface of each chamber only via ion channels or transporters in the biological membrane.

- 5      24. A high throughput screen according to claim 23 wherein the wells are provided by a multiwell plate.
25. A high throughput screen according to claim 22 which comprises an array of droplets on a porous substrate.
- 10      26. A high throughput screen according to any one of claims 22 to 25 which comprises a recording head having a single recording electrode capable of being moved to visit each chamber sequentially.
- 15      27. A high throughput screen according to any one of claims 22 to 25 which comprises a recording head having a plurality of recording electrodes arranged in a line.
28. A high throughput screen according to any one of claims 22 to 25 which comprises a recording head having a plurality of recording electrodes arranged in a matrix.
- 20      29. A method of manufacturing the structure of any one of claims 1 to 19 which comprises the steps of selecting a substrate, perforating it, introducing a biological

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membrane to the substrate and sealing each pore with biological membrane.

30. A method according to claim 29 which comprises the steps of simultaneously perforating a substrate and sealing the pores with biological membrane.
31. A method of manufacturing a biological membrane according to claim 20 which comprises the steps of selecting a cell type, evaluating it for ability to form contiguous layers of cells with tight junctions and for low to negligible numbers of voltage gated ion channels, culturing the cells on a substrate and ensuring that a contiguous layer of cells is grown.
32. A method according to claim 31 which includes the step of permeabilizing one surface of the contiguous layer of cells thereby providing access to the interior of the cells.
33. A method according to claim 32 wherein the step of permeabilizing one surface of the contiguous layer of cells is carried out by the step of contacting the surface with an antibiotic selected from the group which comprises amphotericin and nystatin; or detergent selected from the group which comprises digitonin and saponin; or physical disruption using a high voltage



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field; or by enzymatic digestion of a part of the cell membrane using an appropriate enzyme.

34. A method according to any one of claims 31 to 33 which comprises the steps of transfecting cells with cDNA or  
5 cRNA encoding an ion channel of interest and cloning cells expressing the ion channel of interest.
35. A method of manufacturing a substrate according to claim 21 which comprises the steps of shining a laser of preselected focal area, power or time of exposure  
10 at a coverslip to perforate it.
36. A method according to claim 35 which comprises the steps of adjusting the profile, taper or diameter of the pore with a laser.
37. A method according to claim 35 or 36 wherein the laser  
15 source is controlled by an automated stage under control of a computer and inverted optics microscope.
38. A method of manufacturing a substrate according to claim 21 which comprises a non-laser method selected from the group which comprises photo-etching, casting  
20 and physical piercing of the substrate.

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39. A method of screening for the detection or assay of compounds with activity on ion channels or transporters which comprises the steps of placing a biological membrane which expresses ion channels or transporters of interest in contact with test compound in physiological solution or non-physiological solution comprising dimethyl sulphoxide and measuring the resistance or impedance of the biological membrane under the influence of test compound.
40. A method according to claim 39 wherein ion channel activity is monitored by trans-epithelial resistance measurements (TERM) across an intact cell layer.
41. A method according to claim 39 which comprises the step of permeabilizing a contiguous cell layer to provide access to the interior of the cells permitting intracellular voltage and current measurements to be made.
42. A method according to claim 41 wherein a contiguous cell layer is permeabilized by antibiotic selected from the group which comprises amphotericin and nystatin; or detergent selected from the group which comprises digitonin and saponin; or physical disruption using a high voltage field; or by enzymatic digestion of a part of the cell membrane using an appropriate enzyme

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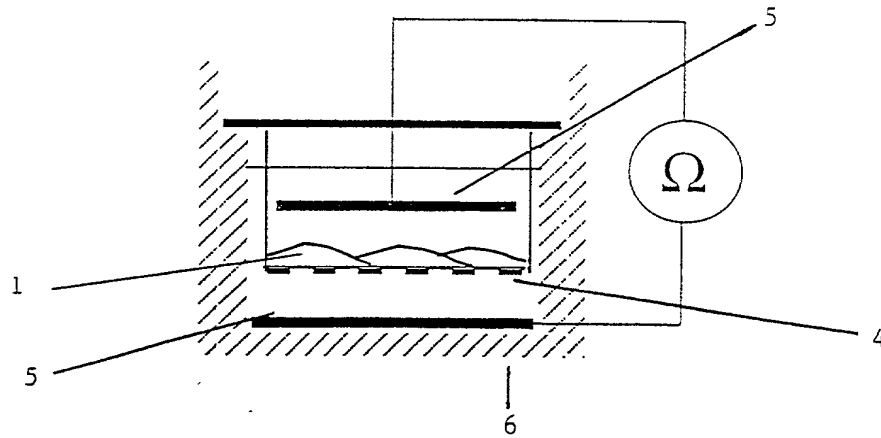
thereby permitting intracellular voltage or current measurements to made.

43. A method according to any one of claims 39 to 42 which includes the step of multiplexing up to 384 recording elements to a data acquisition system utilizing multiple voltage-clamp amplifiers.
44. A method according to any one of claims 39 to 43 which comprises the step of placing an array of droplets having ion channels or transporters of interest therein on a porous substrate and screening test compounds for activity on the ion channels or transporters.
45. A method according to any one of claims 39 to 43 which comprises the step of placing biological membrane having ion channels or transporters of interest and test compounds in physiological solution, or non-physiological solution comprising dimethyl sulphoxide, in a plurality of chambers and screening the test compounds for activity on the ion channels or transporters.
46. A method according to any of claims 21, 30 and 35 to 38 wherein, subsequent to pore formation, the substrate is exposed to localised heat and/or to electrical

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plasma in order to impart an appropriate raised level of smoothness to the pore(s).

A)



B)

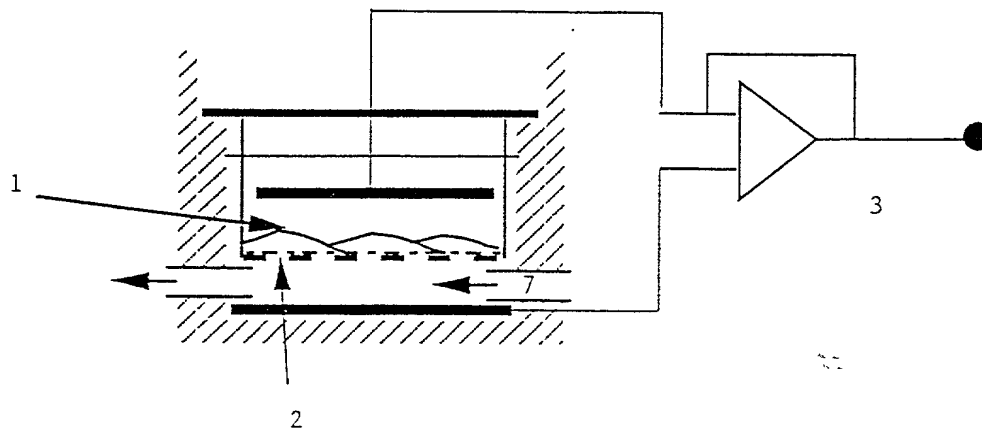
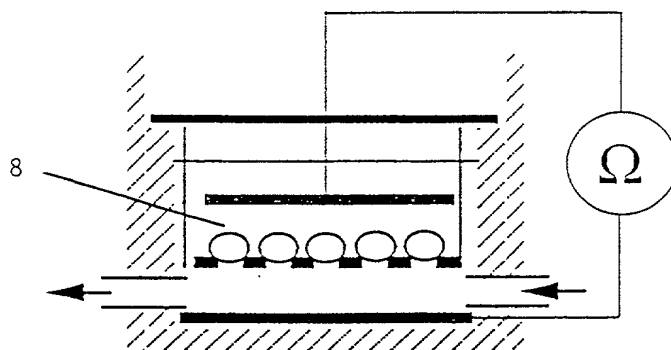


FIGURE 1

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A)



B)

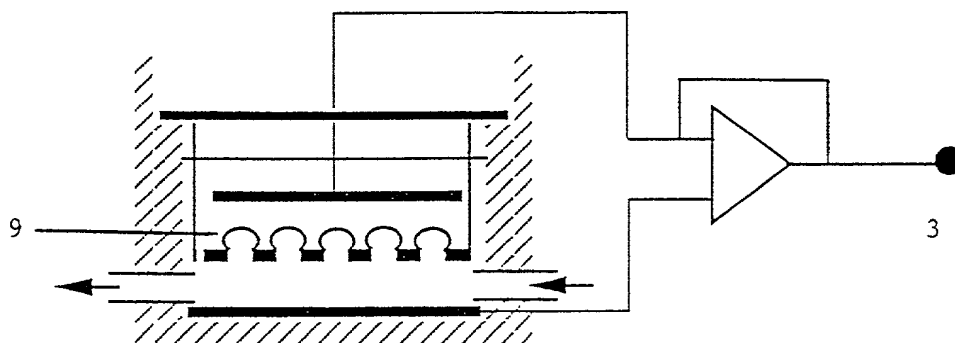


FIGURE 2

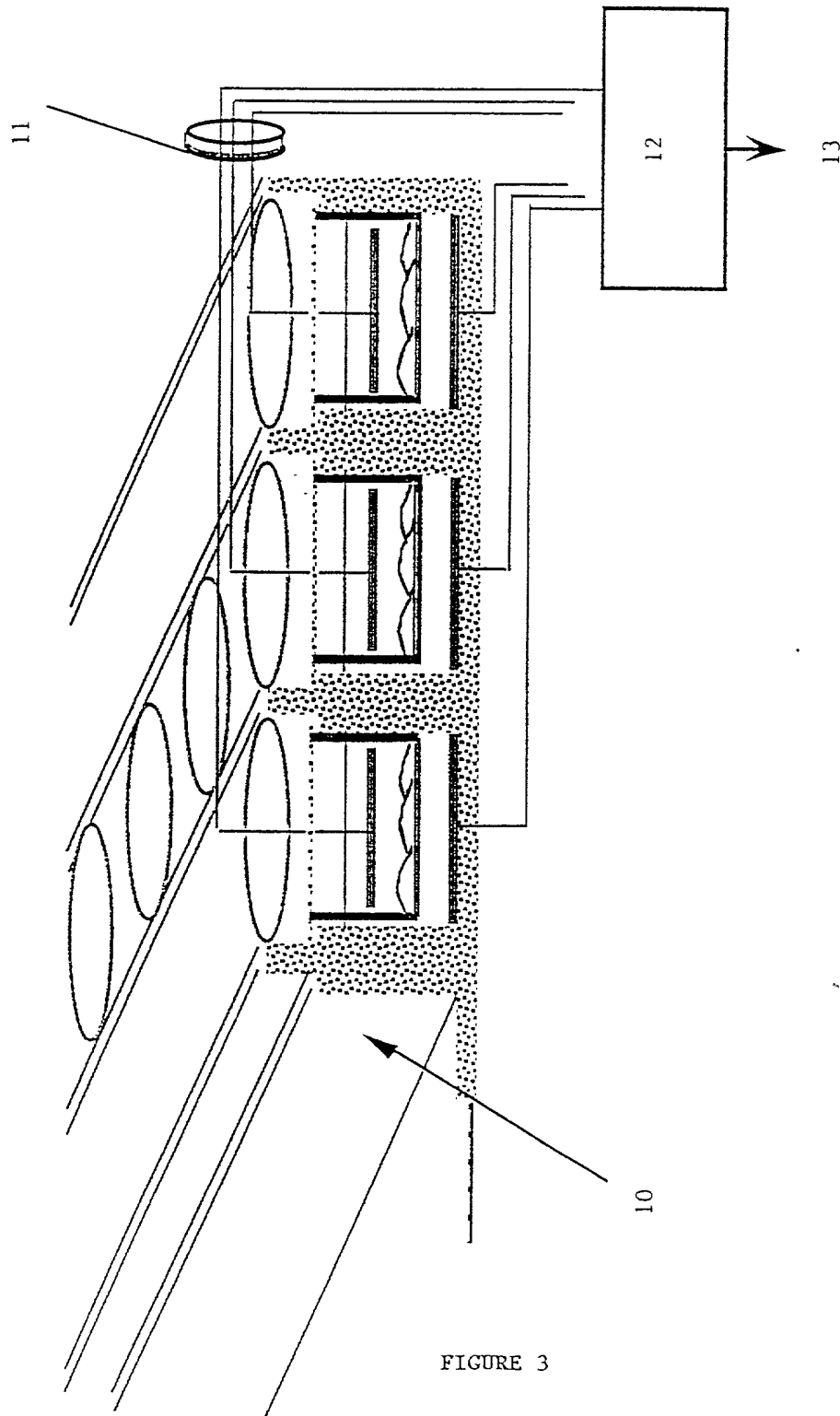


FIGURE 3

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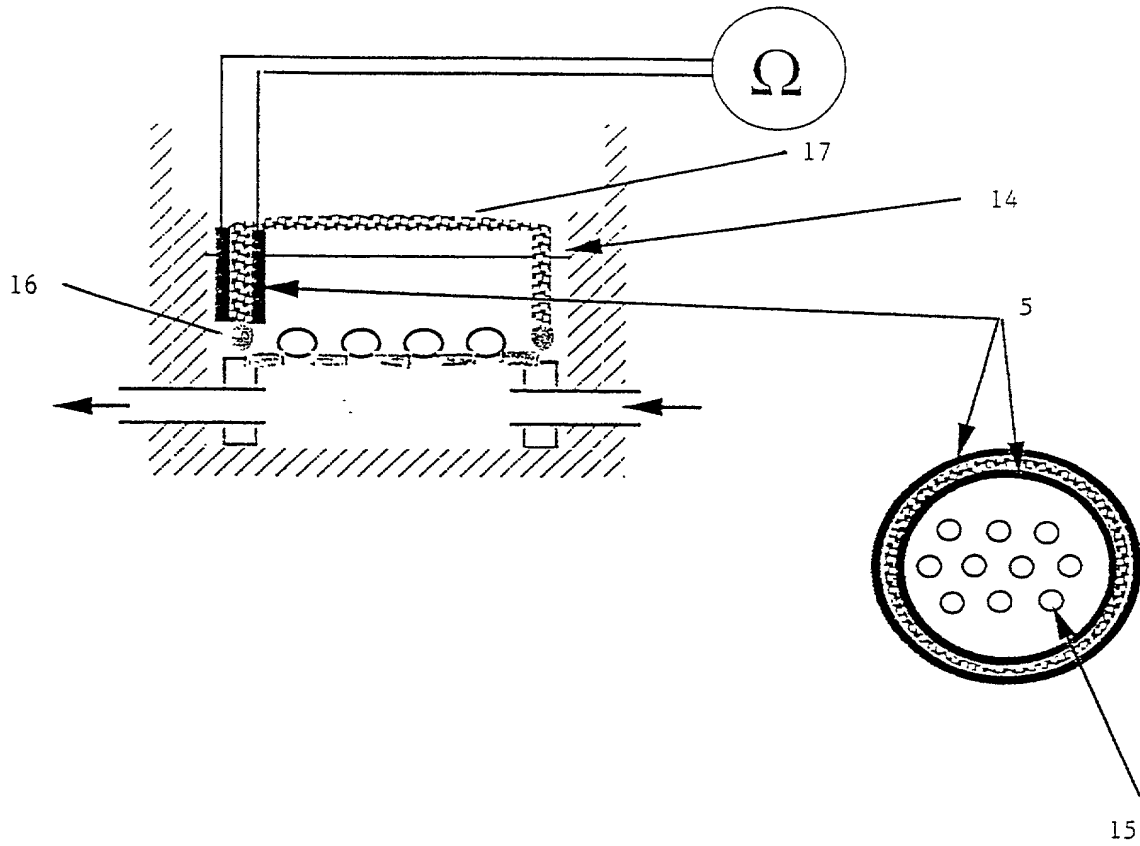


FIGURE 4



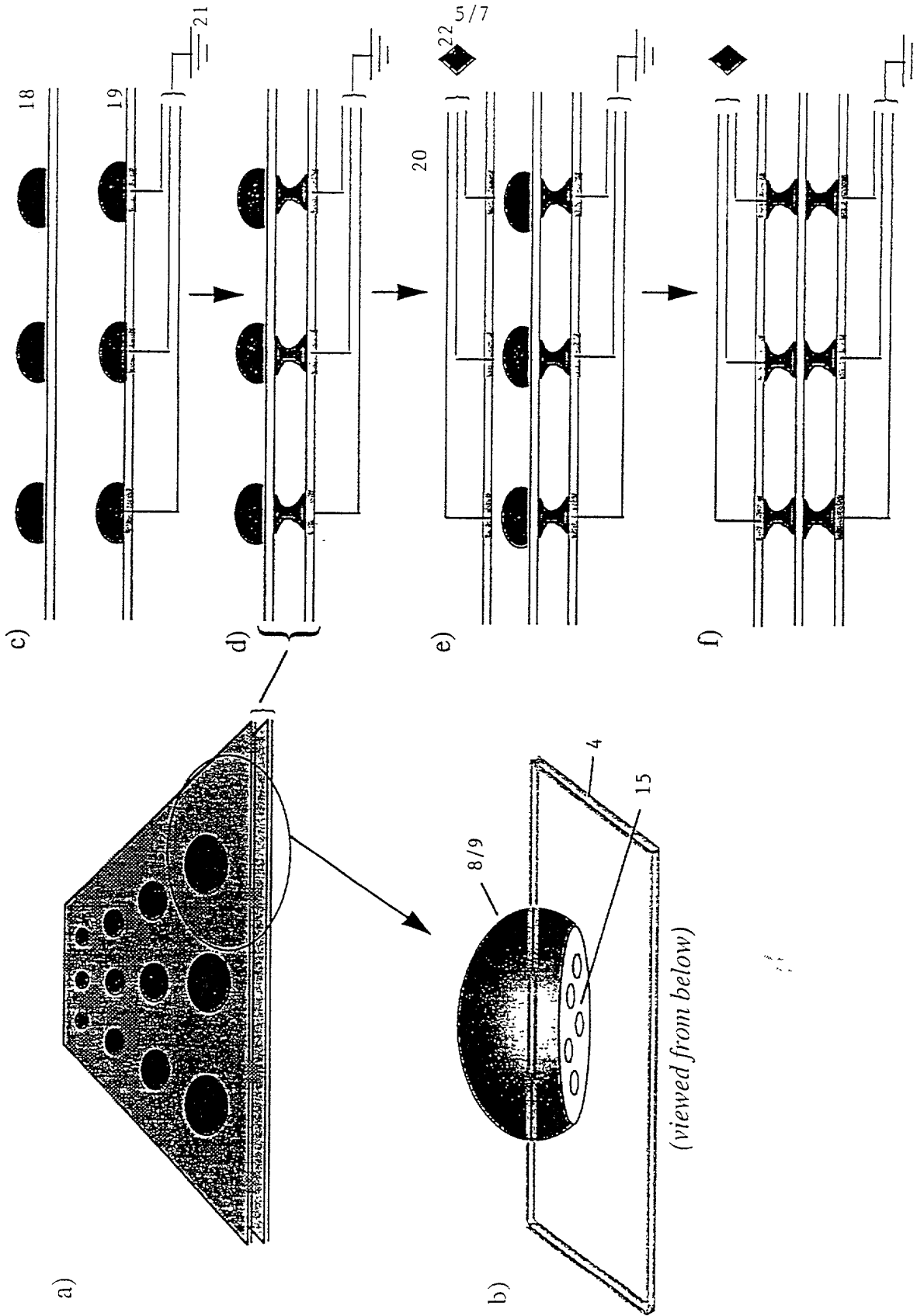


FIGURE 5

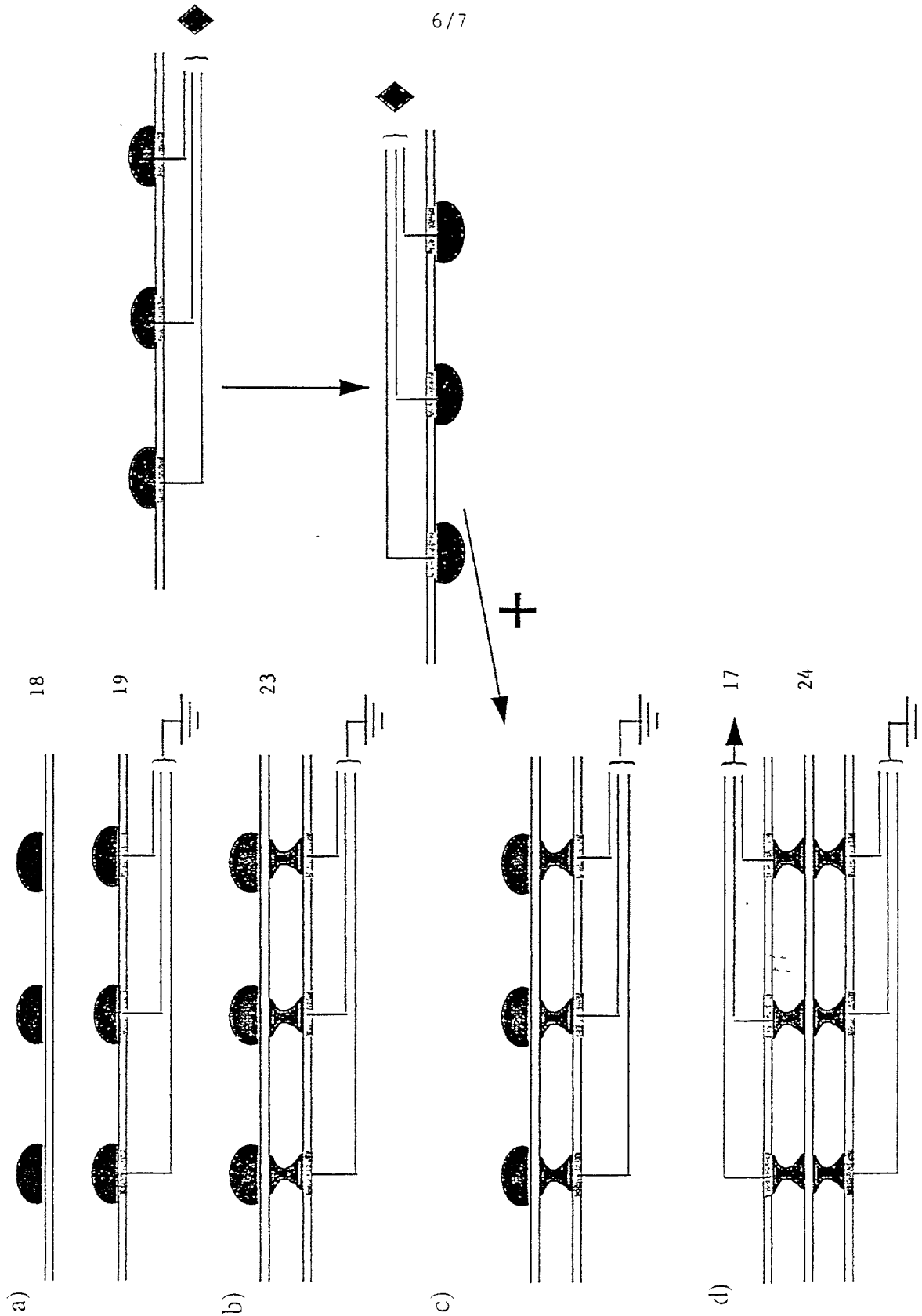


FIGURE 6

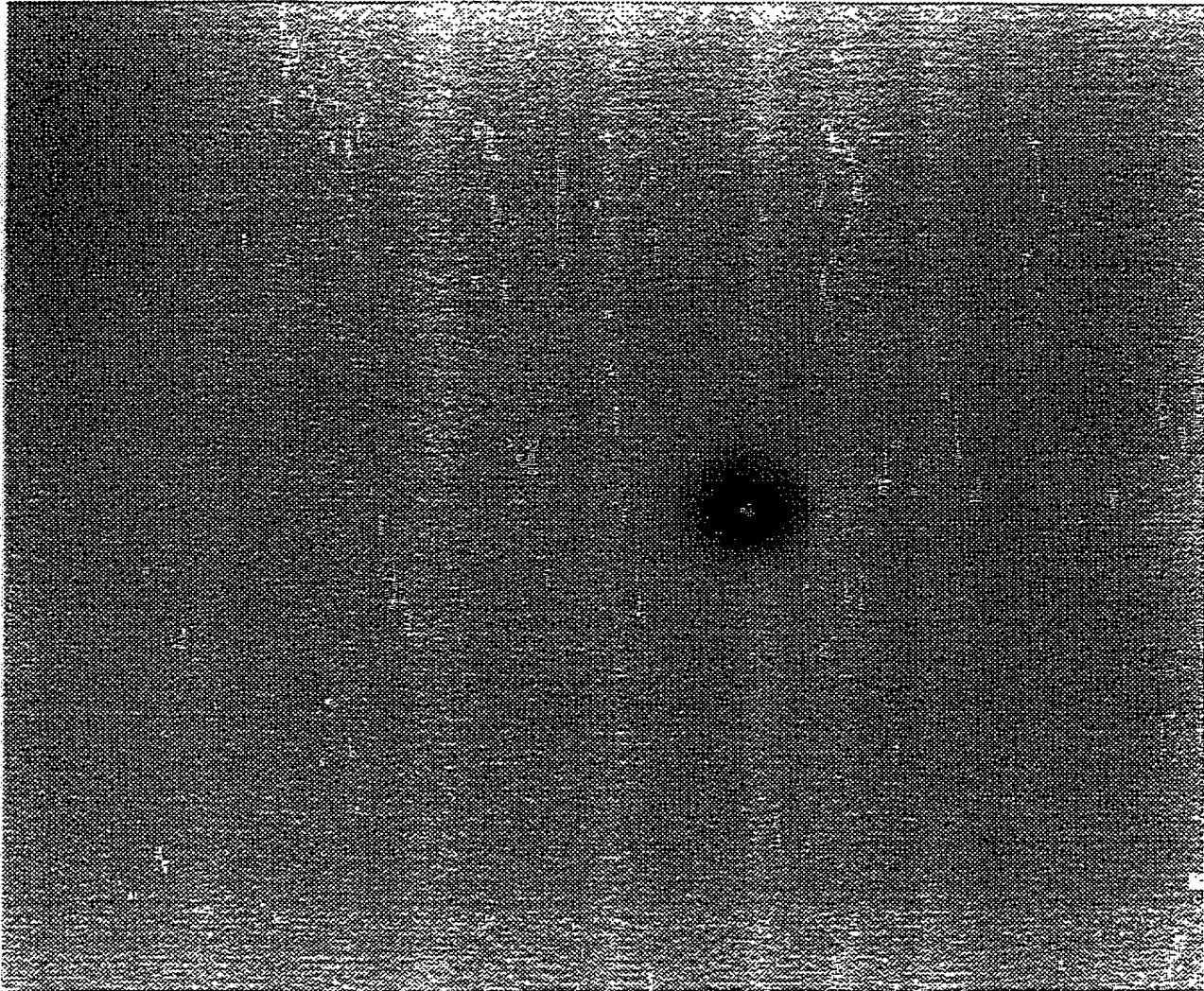


Figure 7

## DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## HIGH THROUGHPUT SCREEN

the specification of which:

is attached hereto; or

was filed as United States Application Serial No. 09/719,236  
on December 11, 2000, and was amended on \_\_\_\_\_  
(if applicable); or

was filed as PCT International Application Number PCT/GB99/01871  
on June 14, 1999 and was amended on \_\_\_\_\_  
(if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s), designating at least one country other than the United States, listed below and have also identified below any foreign application(s) for patent or inventor's certificate, or any PCT international application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
United Kingdom	9812783.0	June 12, 1998	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT international application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.**, Reg. No. 22,540, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilly, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewis, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgujian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Dirk D. Thomas, Reg. No. 32,600; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,616; Bryan C. Diner, Reg. No. 32,409; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheri M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,266; and Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James B. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,629; Allen R. Jensen, Reg. No. 28,224; Lori Ann Johnson, Reg. No. 34,498; and David A. Manspeizer, Reg. No. 37,540 and \_\_\_\_\_.

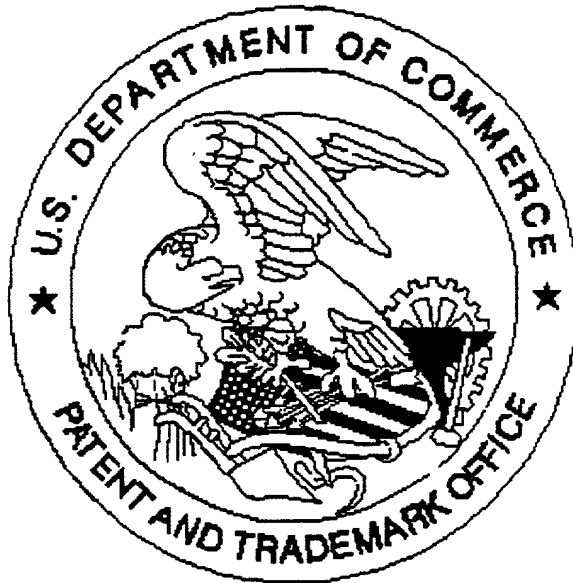
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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